PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY

(Chapter II of the Patent Cooperation Treaty)

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Form PCT/IPEA/416
M30406PCT		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/EP2004/002216	04.03.2004	11.04.2003

International Patent Classification (IPC) or national classification and IPC
C12N15/10

Applicant
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1. This report is the international preliminary examination report, established by this International Preliminary Examining Authority under Article 35 and transmitted to the applicant according to Article 36.
2. This REPORT consists of a total of 10 sheets, including this cover sheet.
3. This report is also accompanied by ANNEXES, comprising:
   a. ☒ *sent to the applicant and to the International Bureau)* a total of 4 sheets, as follows:
      ☒ sheets of the description, claims and/or drawings which have been amended and are the basis of this report and/or sheets containing rectifications authorized by this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions).
      ☐ sheets which supersede earlier sheets, but which this Authority considers contain an amendment that goes beyond the disclosure in the international application as filed, as indicated in item 4 of Box No. I and the Supplemental Box.
   b. ☐ *(sent to the International Bureau only)* a total of (indicate type and number of electronic carrier(s)) , containing a sequence listing and/or tables related thereto, in computer readable form only, as indicated in the Supplemental Box Relating to Sequence Listing (see Section 802 of the Administrative Instructions).

4. This report contains indications relating to the following items:

☒	Box No. I	Basis of the opinion
☐	Box No. II	Priority
☐	Box No. III	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
☐	Box No. IV	Lack of unity of invention
☒	Box No. V	Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
☐	Box No. VI	Certain documents cited
☐	Box No. VII	Certain defects in the international application
☒	Box No. VIII	Certain observations on the international application

Date of submission of the demand	Date of completion of this report
21.07.2004	27.06.2005
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized Officer Pilat, D Telephone No. +49 89 2399-8668

INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY

International application No.
PCT/EP2004/002216

Box No. I Basis of the report

1. With regard to the **language**, this report is based on the international application in the language in which it was filed, unless otherwise indicated under this item.

   ☐ This report is based on translations from the original language into the following language , which is the language of a translation furnished for the purposes of:
   - ☐ international search (under Rules 12.3 and 23.1(b))
   - ☐ publication of the international application (under Rule 12.4)
   - ☐ international preliminary examination (under Rules 55.2 and/or 55.3)

2. With regard to the **elements*** of the international application, this report is based on *(replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report)*:

Description, Pages	
1-17	as originally filed
Claims, Numbers	
1-21, 24-27	filed with the demand
Drawings, Sheets	
1/5-5/5	as originally filed

   ☐ a sequence listing and/or any related table(s) - see Supplemental Box Relating to Sequence Listing

3. ☒ The amendments have resulted in the cancellation of:
   - ☐ the description, pages
   - ☒ the claims, Nos. 22,23
   - ☐ the drawings, sheets/figs
   - ☐ the sequence listing *(specify)*:
   - ☐ any table(s) related to sequence listing *(specify)*:

4. ☐ This report has been established as if (some of) the amendments annexed to this report and listed below had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).
   - ☐ the description, pages
   - ☐ the claims, Nos.
   - ☐ the drawings, sheets/figs
   - ☐ the sequence listing *(specify)*:
   - ☐ any table(s) related to sequence listing *(specify)*:

* *If item 4 applies, some or all of these sheets may be marked "superseded."*

INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY

International application No.
PCT/EP2004/002216

Box No. V Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	
	No:	Claims	1-4,6-15,17-21,24-27
Inventive step (IS)	Yes:	Claims	
	No:	Claims	5,16
Industrial applicability (IA)	Yes:	Claims	1-21,24-27
	No:	Claims	

2. Citations and explanations (Rule 70.7):

see separate sheet

Box No. VIII Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

1. The documents cited in the search report are referred to in this communication

D1: WO 01/60975 A (BOONE CHARLES ;BUSSEY HOWARD (CA); JIANG BO (CA); ROEMER TERRY (CA) 23 August 2001 (2001-08-23)

D2: BOCKAMP ERNESTO ET AL: "Of mice and models: Improved animal models for biomedical research." PHYSIOLOGICAL GENOMICS, vol. 11, 20 January 2003 (2003-01-20), pages 115-132, XP009014519 ISSN: 1094-8341

D3: GOTTHARDT MICHAEL ET AL: "Conditional expression of mutant M-line titins results in cardiomyopathy with altered sarcomere structure." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 278, no. 8, 21 February 2003 (2003-02-21), pages 6059-6065, XP002249070 ISSN: 0021-9258

D4: POOK M A ET AL: "Rescue of the Friedreich's ataxia knockout mouse by human YAC transgenesis." NEUROGENETICS. ENGLAND OCT 2001, vol. 3, no. 4, October 2001 (2001-10), pages 185-193, XP002249071 ISSN: 1364-6745

D5: HAMILTON-WILLIAMS EMMA E ET AL: "Transgenic rescue implicates beta2-microglobulin as a diabetes susceptibility gene in nonobese diabetic (NOD) mice." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES, vol. 98, no. 20, 25 September 2001 (2001-09-25), pages 11533-11538, XP002249072 September 25, 2001 ISSN: 0027-8424

D6: MONANI UMRAO R ET AL: "A transgene carrying an A2G missense mutation in the SMN gene modulates phenotypic severity in mice with severe (type I) spinal muscular atrophy." JOURNAL OF CELL BIOLOGY, vol. 160, no. 1, 6 January 2003 (2003-01-06), pages 41-52, XP002249073 ISSN: 0021-9525

D7: WUTZ A ET AL: "Non-imprinted Igf2r expression decreases growth and rescues the Tme mutation in mice." DEVELOPMENT (CAMBRIDGE), vol. 128, no. 10, May 2001 (2001-05), pages 1881-1887, XP002249074 ISSN: 0950-1991

D8: GANTOIS I ET AL: "Restoring the phenotype of fragile X syndrome: Insight from the mouse model." CURRENT MOLECULAR MEDICINE (HILVERSUM), vol. 1, no. 4, September 2001 (2001-09), pages 447-455, XP009014502 September, 2001 ISSN: 1566-5240

D9: US-B1-6 291 245 (SCHANTZ CHRISTIAN ET AL) 18 September 2001 (2001-09-18)

Ad Section V :Reasoned statement under Rule 66.2(a)(ii); citations and explanations supporting such statement

The present application is directed to method to study mutations in an organism or cell by circumventing lethality or adverse effects of said mutations. The present application is in addtion directed to a conditionally inducible site-directed mutant cell.

As disclosed in the present application at page 3, the smallest modification that can be introduced into genomic region of choice using conditional knock-out technology is the deletion of a single exon. Mutations of single bases that causes a lethal phenotype cannot be temporally or locally regulated. In addition for the production of multiple inducible knock-outs of a single gene more than one conditionally inducible targeting vector is required.
The information derivable from this passage focuses on some advantages of the method disclosed (e.g. Mutations can be temporally and locally regulated, in order to provide an adult organism that would be present for analysis; ...production of multiple inducible knock-outs of a single gene may be performed in a more convenient manner; The present system lacks flexibility p.3 2nd full §).
The present application discloses the use of a conditional abandoning to go from wild-type to knock-out, while the published data use the gene rescue to go from knock-out to wild-type (see application p.5 3§).

2 **Novelty (Article 33 (2) PCT)**

2.0 Claim 1 relates to a product per se. The conditionally inducible site-directed mutant cell has been interpreted as being a conditionally inducible site-directed mutated gene containing cell. Claim 1 does not refer to a method including a site-directed mutagenesis and does not refer to a method including an non-mutated allele which can be selectively excised either.
Claim 1 uses an open-ended transition word "comprising" both for the content of the cell and for the mutated allele characterizing said cell. A mutated allele of a gene "comprising" a mutation at the exon or sub-exon level is any allele having at least a mutation at the exon or sub-exon level. An allele having a deletion of more than one exon falls under the scope of this definition. The list of some possible type of

mutations, which is endless due to the expression "and the like", is not limiting either for the mutated gene. The features following the expression "such as" must be considered as entirely optional (see also PCT Guidelines PART II 5.40).
With regard to the rescue allele, the clarity objection raised below under item VIII applies.

2.1 WO0160975 discloses a strain of diploid fungal cells comprising modified alleles of a gene, wherein the first allele of the gene is inactivated by recombination using a gene disruption cassette comprising a nucleotide sequence encoding an expressible selectable marker ; and the expression of the second allele of the gene is regulated by a heterologous promoter that is operably linked to the coding region of the second allele of the gene. Said diploid fungal cells further comprises a nucleotide sequence encoding a transactivation fusion protein that is expressible in the diploid fungal cell, said transactivation fusion protein comprising a DNA binding domain and a transcription activation domain ; and wherein the heterologous promoter in the promoter replacement fragment comprises at least one copy of a nucleotide sequence which is bound by the DNA binding domain of the transactivation fusion protein, such that binding of the transactivation fusion protein increases transcription from the heterologous promoter (see claims 10, 11).

2.2 WO0160975 discloses a collection of strains each comprising the modified alleles of different gene, wherein each gene is essential for the growth and/or survival of the cells are also claimed (see claim 15).

2.3 WO0160975 describes also a method for constructing a strain of diploid fungal cells in which both alleles of a gene are modified, the method comprising the steps of : (a) modifying a first allele of a gene in diploid fungal cells by recombination using a gene disruption cassette comprising a first nucleotide sequence encoding an expressible selectable marker, thereby providing heterozygous diploid fungal cells in which the first allele of the gene is inactivated ; and (b) modifying the second allele of the gene in the heterozygous diploid fungal cells by recombination using a promoter replacement fragment comprising a second nucleotide sequence encoding a heterologous promoter, such that expression of the second allele of the gene is regulated by the heterologous promoter (see claim 1). This last step corresponds to a

transfection or infection of the cell with a genetic construct comprising at least attachment of antisense oligonucleotides.
In one embodiment, such mutant cells are cultured under conditions where the second allele of the modified gene is substantially not expressed. The viability or pathogenicity of the cells are then determined. The resulting loss of viability or exhibition of a severe growth defect indicates that the gene that is modified in the mutant cells is essential to the survival of a pathogenic fungus (see p.5 lines 31-35). In another embodiment, the second allele of the target gene may be substantially underexpressed to provide cells with enhanced sensitivity to compounds active against the gene product expressed by the modified allele. Alternatively, the second allele may be substantially overexpressed to provide cells with increased resistance to compounds active against the gene product expressed by the modified allele of the target gene (see p.6 line 8-12).

In summary the method of the WO0160975, as applied to a diploid cell involves two steps : (i) gene replacement resulting in disruption of the coding and/or non-coding region (s) of one wild type allele by insertion, truncation, and/or deletion, and (ii) conditional expression of the remaining wild type allele via promoter replacement or conditional protein instability (Fig. 2).

2.4 Finally, WO0160975 is equally directed toward methods for modulating expression of an essential gene which has been identified by the methods described supra, in which an antisense RNA molecule, which inhibits translation of mRNA transcribed from an essential gene, is expressed from a regulatable promoter. In one aspect of this embodiment, the antisense RNA molecule is expressed in a GRACE strain of Candida albicans or another GRACE strain constructed from another diploid pathogenic organism (see p.56 lines 17-23). The same approach is suggested for ribozymes (see p.60 lines 24-32).

No structural information characterizing the claimed sites are given in claim 2. They may be of any structural composition. They are present in the modified cell disclosed in D1.

The product of claim 3 attempts to further characterize said rescue allele in that it

comprises a conditionally inducible gene or genetic construct which either directly or via its expression product inhibit the function of any non-mutated copy of said mutated allele. The function is not identified. Thus any function is acceptable. The activity of exercising either directly or via its expression product an inhibitory effect must be at best interpreted for a product claim as being capable of eliciting this effect. The cell disclosed in D1, in the presence of tetracycline, is capable to directly inhibit the function of being expressed of the rescue allele (see D1, Fig.3). Furthermore, D1 proposes alternative methods of conditional expression like the introduction of a ubiquination signal in the wild type remaining gene or by introducing lexA operator elements in the promoter (see p.22 §5.2.3). Thus, claim 3 lacks novelty.

2.5 In view of this teaching, claims 1-4, 6-11 and 12-15,17-21,24-27 lack novelty.

2.6 Bockamp et al. reviews several methods for producing animal models. In particular a switchable gene knockout method. Said method has been applied to *Ednrb* gene. The spatiotemporal regulation has been achieved, in that a tet-inducible transactivator (tTA) was integrated in the endogenous *Ednrb* locus. This substitute and inactivates the first allele (effector). A conditional *Ednrb* responder knock-in cassette replaces the second allele.
Thus, first expression of the tet-dependent transactivator mirrors the expression of the endogenous *Ednrb* gene and second the knock-in leads to the complete inactivation of the other *Ednrb* allele (knock-in effector) when not rescued with DOX (see p.126 col.1-2; Fig.7). The review focus also on additional developments, using for example RNAi based approaches (see p.128 col.2 lines 6-14).

2.7 In view of this document, claims 1-4, 6-11 and 12-15,17-21,24-27 lack novelty.

2.8 US6291245 discloses an auxotrophic microorganism. In particular, it relates to a prokaryotic auxotrophic host cell which contains a vector according to the invention wherein this host cell has a mutation (deletion) in a gene which is complemented by the selection marker gene of the expression vector and the deletion or mutation has the effect that no functional product of the said host cell gene is expressed. The deletion is preferably located in an essential chromosomal gene which corresponds to the auxotrophy marker gene of the expression vector.

2.9 The same conclusion may be drawn based on the transgenic rescue of described β2-M-deficient NOD mice with various β2-M isoforms in Hamilton-Williams et al. (see p.11533 col.2 last two sentence of the introduction; and p.11534 col.1 transgenic mice genotype). The same applies based on the content of Wutz et al. (see p.1884, col.2 "Complementation of the Igf2r null allele ..." and Table 1), Monani et al. (see abstract and of the transgenic mice null mutant genotype), and Pook e al (see p.186 last § of the introduction; p.189 col.2), and Gantois (see abstract, p.449 "Attempt to rescue the fragile X knock-out mutations (see also Fig.2).

These document anticipates also one or more of the above cited claims.

3. Inventive step (Article 33(3) PCT)

3.1 The mere combination of the prior art previous techniques with a particular gene, titin, which is equally known in the art (see Gotthardt et al.), does not seem to involve an inventive step. Thus, claims 5 and 16 lack an inventive step.

Ad Section VIII : Certain observations on the international application.

4 Clarity (Article 6 PCT)

4.1 Claim 1 and claims dependent thereon are product claims. They need to be characterized by means of essential features which unambiguously identify the product claimed.
In the light of this rationale, claim 1 must be read as being "a conditionally inducible site directed mutant cell, comprising a mutated allele of a gene and a rescue allele of said mutated gene, wherein said mutation is said mutated allele of said gene interferes with the survival and/or cause an adverse phenotype.
The process features in a) "that was introduced ..." and b) "that can be inactivated" do not provide features which would further characterize the product claimed.

4.2 Features following the expression "such as", "e.g.", " in particular", "optionally" must be considered as entirely optional (see also PCT Guidelines PART II 5.40). Claims

2,6,7,9,11,12, 13,17,19,21,25,27 are concerned.

4.3 It is reminded that any prior art gene may be conditionally inactivated, even if it has not yet been in the prior art inactivated, by means of an antisense or ribozyme etc...

4.4 A multiply mutated allele of a gene is a product by process feature. It is interpreted as meaning that the an allele of a gene has been mutated at least twice. Insofar as claim 4 refers to a product, the end-product obtained by this process is the only relevant subject-matter. Thus a cell as described in D1 falls under the scope of protection of claim 4. The cell of D1 are considered to be obtainable by the introduction of multiple mutations.

M30406PCT
Max-Delbrück-Centrum für molekulare Medizin

Patent Claims (amended)

1. A conditionally inducible site-directed mutant cell, comprising
   a) a mutated allele of a gene comprising a mutation at the exon or sub-exon level, such as a deletion, point mutation, insertion, inversion, and the like, and
   b) a rescue allele of said mutated gene in the form of a gene or genetic construct that can be conditionally inactivated,

   wherein said mutation in said mutated allele of said gene interferes with survival and/or causes an adverse phenotype.

2. The conditionally inducible site-directed mutant cell according to claim 1, wherein said rescue allele and/or its transcription product(s) comprises recombination target sites, such as lox or FRT sites, sites for the attachment of antisense oligonucleotides, such as DNA, PNA and/or RNA-oligonucleotides, sites for ribozyme activities, and or sites that interfere with specific siRNA for expression.

3. The conditionally inducible site-directed mutant cell according to claim 1 or 2, wherein said rescue allele comprises a conditionally inducible gene or genetic construct which either directly or via its expression product inhibits the function of any non-mutated copy of said mutated allele.

4. The conditionally inducible site-directed mutant cell according to any of claims 1 to 3, containing multiple mutated alleles of genes and/or a multiply mutated allele of a gene together with their suitable rescue allele(s).

5. The conditionally inducible site-directed mutant cell according to any of claims 1 to 4, wherein said allele encodes for titin.

6. The conditionally inducible site-directed mutant cell according to any of claims 1 to 5, wherein said interference with survival and/or adverse phenotype is selected from temporal and/or local phenotypes, such as cell cycle-specific, cell-type specific, tissue-

specific, protein-expression specific, tissue-development specific, organ-specific, organ-development-specific and/or embryonic lethal phenotypes.

7. The conditionally inducible site-directed mutant cell according to any of claims 1 to 6, which is selected from a prokaryotic cell, a eukaryotic cell, a diploid cell, a plant cell, a mammalian cell, a nematode cell, a fish cell, an insect cell, and, in particular, a non-human stem-cell.

8. A conditionally inducible site-directed mutant cell culture, tissue, organ, or non-human embryo, comprising a cell according to any of claims 1 to 7.

9. A conditionally inducible site-directed mutant non-human organism, in particular a genetically deficient or Knock-out-mammal, -rodent, -nematode, -fish, -plant or -insect, comprising a cell according to any of claims 1 to 7 or a culture, tissue or organ according to claim 8.

10. The conditionally inducible site-directed mutant non-human organism according to claim 9, containing multiple mutated alleles of genes and/or a multiply mutated allele of a gene together with their suitable rescue allele(s).

11. The conditionally inducible site-directed mutant non-human organism according to claim 9 or 10, wherein said interference with survival and/or adverse phenotype is selected from temporal and/or local phenotypes, such as cell cycle-specific, cell-type specific, tissue-specific, tissue-development specific, protein-expression specific, organ-specific, organ-development-specific and/or embryonic lethal phenotypes.

12. A method for producing an inducible site-directed mutant cell capable of conditional gene rescue, comprising
    a) introducing in a target cell a mutated allele of a gene to be mutated by using a suitable mutagenesis technique,
    b) introducing in said target cell a rescue allele of said gene that can be conditionally inactivated, and
    c) optionally, cultivating said target cell under conditions that allow for a selection of cells that contain both the mutated allele and the rescue allele of said gene,

wherein said mutation in said mutated allele of said gene interferes with survival and/or causes an adverse phenotype, and

wherein said suitable mutagenesis technique comprises introducing a mutation at the exon or sub-exon level, such as a deletion, point mutation, insertion, inversion, and the like, preferably by using a suitable mutagenesis technique employing a vector system, irradiation, random integration of foreign DNA, site specific recombination, homologous recombination, and/or chemical mutagenesis.

13. The method according to claim 12, wherein introducing said rescue allele comprises transfection or infection of the cell with a rescue allele gene or genetic construct comprising recombination target sites, e.g. lox or FRT sites, sites for the attachment of antisense oligonucleotides, e.g. DNA, PNA and/or RNA-oligonucleotides, sites for ribozyme activities, and or sites that interfere with specific siRNA for expression.

14. The method according to claim 12 or 13, wherein introducing said rescue allele comprises transfer of a conditionally inducible gene or genetic construct into the cell, which either directly or via its expression product inhibits the function of any non-mutated copy of said mutated allele.

15. The method according to any of claims 12 to 14, wherein a tissue specific rescue allele and/or mutated allele is introduced.

16. The method according to any of claims 12 to 15, wherein said allele encodes for titin.

17. The method according to any of claims 12 to 16, wherein said cell is selected from a prokaryotic cell, a eukaryotic cell, a diploid cell, a plant cell, a mammalian cell, a fish cell, a nematode cell, an insect cell, and, in particular, a non-human stem-cell.

18. The method according to any of claims 12 to 17, comprising the introduction of multiple mutated alleles of genes and/or a multiply mutated allele of a gene together with their suitable rescue allele(s).

19. The method according to any of claims 12 to 18, wherein said interference with survival and/or adverse phenotype is selected from temporal and/or local phenotypes, such as cell

cycle-specific, cell-type specific, tissue-specific, tissue-development specific, organ-specific, organ-development-specific and/or embryonic lethal phenotypes.

20. The method according to any of claims 12 to 19, further comprising
    d) conditionally inactivating said rescue allele of said gene to be mutated by using a suitable inactivation technique.

21. The method according to claim 21, wherein conditionally inactivating said rescue allele of said gene to be mutated by using a suitable inactivation technique comprises a technique selected from site directed recombination, such as cre/lox or Flp/FRT inactivation, antisense inactivation using oligonucleotides, e.g. DNA, PNA and/or RNA-oligonucleotides, RNA-interference, such as ribozyme activity inactivation, siRNA expression-inactivation, inactivation of the gene product (protein) and/or its activity and/or inducible inactivation of the non-mutated allele, such as through antibodies, inactivation of the activity of a fusion protein or induced proteolysis.

24. The method according to any of claims 13 to 23, wherein said method is performed in vivo or in vitro.

25. The method according to any of claims 13 to 24, wherein said cell is present in a tissue, organ, non-human embryo or non-human organism, in particular a mammal, rodent, nematode, fish, plant, or insect.

26. A method for the production of an inducible site-directed non-human mutant-organism capable of conditional gene rescue, comprising
    a) generating an inducible site-directed mutant cell according to the method according to any of claims 13 to 24, and
    b) generating a non-human mutant organism comprising said mutant cell.

27. An inducible site-directed non-human mutant-organism, produced according to claim 26, in particular a mammal, nematode, rodent, fish, plant, or insect.